T HELPER CELL EPITOPES

RELATED APPLICATION DATA

This application is a divisional patent application of USSN 09/890,650 filed March 22, 2002 which is a 371 of International Patent Application No. PCT/AU00/00070 filed on February 7, 2000, which claims benefit of foreign priority under 35 USC §119 from Australian Patent Application No. PP8533 filed on February 5, 1999 and Australian Patent Application No. PQ2013 filed on August 4, 1999.

FIELD OF THE INVENTION

The present invention relates to T helper cell epitopes derived from Canine Distemper Virus (CDV). The present invention relates to compositions including at least one T helper cell epitope and optionally B cell epitopes and/or CTL epitopes.

BACKGROUND OF THE INVENTION

For any peptide to be able to induce an effective antibody response it must contain particular sequences of amino acids known as epitopes that are recognised by the immune system. In particular, for antibody responses, epitopes need to be recognised by specific immunoglobulin (Ig) receptors present on the surface of B lymphocytes. It is these cells which ultimately differentiate into plasma cells capable of producing antibody specific for that epitope. In addition to these B cell epitopes, the immunogen must also contain epitopes that are presented by antigen presenting cells (APC) to specific receptors present on helper T lymphocytes, the cells which are necessary to provide the signals required for the B cells to differentiate into antibody producing cells.

In the case of viral infections and in many cases of cancer, antibody is of limited benefit in recovery and the immune system responds with cytotoxic T cells (CTL) which are able to kill the virus-infected or cancer cell. Like helper T cells, CTL are first activated by interaction with APC bearing their specific peptide epitope presented on the surface, this time in association with MHC class I rather than class II molecules. Once activated the CTL can engage a target cell bearing the same peptide/class I complex and cause its lysis. It is also becoming apparent that helper T cells play a role in this process; before the APC is capable of activating the CTL it must first receive signals from the helper T cell to upregulate the expression of the necessary costimulatory molecules.

Helper T cell epitopes are bound by molecules present on the surface of APCs that are coded by class II genes of the major histocompatibility complex (MHC). The complex of the class II molecule and peptide epitope is then recognised by specific T-cell

receptors (TCR) on the surface of T helper lymphocytes. In this way the T cell, presented with an antigenic epitope in the context of an MHC molecule, can be activated and provide the necessary signals for the B lymphocyte to differentiate. Traditionally the source of helper T cell epitopes for a peptide immunogen is a carrier protein to which peptides are covalently coupled but this coupling procedure can introduce other problems such as modification of the antigenic determinant during the coupling process and the induction of antibodies against the carrier at the expense of antibodies which are directed toward the peptide (Schutze, M. P., Leclerc, C. Jolivet, M. Audibert, F. Chedid, L. Carrier-induced epitopic suppression, a major issue for future synthetic vaccines. J Immunol. 1985, 135, 2319-2322; DiJohn, D., Torrese, J. R. Murillo, J. Herrington, D.A. *et al.* Effect of priming with carrier on response to conjugate vaccine. The Lancet. 1989, 2, 1415-1416). Furthermore, the use of irrelevant proteins in the preparation introduces issues of quality control. The choice of appropriate carrier proteins is very important in designing peptide vaccines and their selection is limited by factors such as toxicity and feasibility of their large scale production. There are other limitations to this approach including the size of the peptide load that can be coupled and the dose of carrier that can be safely administered (Audibert, F. a. C., L. 1984. Modern approaches to vaccines. Molecular and chemical basis of virus virulence and immunogenicity., Cold Spring Harbor Laboratory, New York.). Although carrier molecules allow the induction of a strong immune response they are also associated with undesirable effects such as suppression of the anti-peptide antibody response (Herzenberg, L. A. and Tokuhisa, T. 1980. Carrier-priming leads to hapten-specific suppression. Nature 285:664; Schutze, M. P., Leclerc, C., Jolivet, M., Audibert, F., and Chedid, L. 1985. Carrier-induced epitopic suppression, a major issue for future synthetic vaccines. J Immunol 135:2319; Etlinger, H. M., Felix, A. M., Gillessen, D., Heimer, E. P., Just, M., Pink, J. R., Sinigaglia, F., Sturchler, D., Takacs, B., Trzeciak, A., and et, a. 1988. Assessment in humans of a synthetic peptide-based vaccine against the sporozoite stage of the human malaria parasite, Plasmodium falciparum. J Immunol 140:626).

In general then, an immunogen must contain epitopes capable of being recognised by helper T cells in addition to the epitopes that will be recognised by surface Ig or by the receptors present on cytotoxic T cells. It should be realised that these types of epitopes may be very different. For B cell epitopes, conformation is important as the B cell receptor binds directly to the native immunogen. In contrast, epitopes recognised by T cells are not dependent on conformational integrity of the epitope and consist of short sequences of approximately nine amino acids for CTL and slightly longer sequences, with less restriction on length, for helper T cells. The only requirements for these

epitopes are that they can be accommodated in the binding cleft of the class I or class II molecule respectively and that the complex is then able to engage the T-cell receptor. The class II molecule's binding site is open at both ends allowing a much greater variation in the length of the peptides bound (Brown, J. H., T. S. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger and D. C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364:33) with epitopes as short as 8 amino acid residues being reported (Fahrer, A.M., Geysen, H.M., White, D.O., Jackson, D.C. and Brown,L.E. Analysis of the requirements for class II-restricted T-cell recognition of a single determinant reveals considerable diversity in the T-cell response and degeneracy of peptide binding to I-Ed J. Immunol. 1995. 155: 2849-2857).

Canine distemper virus (CDV) belongs to the subgroup of morbillivirus of paramyxovirus family of negative-stranded RNA viruses. Other viruses which are members of this group are measles virus and rinderpest virus. Development of peptide based vaccines has aroused considerable interest in identification of B and T cell epitopes from sequences of proteins. The rationale for using T cell epitopes from proteins such as the F protein of CDV is that young dogs are inoculated against CDV in early life and will therefore possess helper T cells specific for helper T cell epitopes present on this protein. Subsequent exposure to a vaccine which contains one or more of the epitopes will therefore result in recruitment of existing helper T cells and consequently an enhanced immune response. Such helper T cell epitopes could, however, be administered to unprimed animals and still induce an immune response. The present inventors aimed to identify canine T cell epitopes from the sequence of CDV fusion protein so that these epitopes can then be used in the design of peptide based vaccines, in particular, for the canine and related species.

LHRH (Luteinising hormone releasing hormone) is a ten amino acids long peptide hormone whose sequence is conserved in mammals. It is secreted by the hypothalamus and controls the reproductive physiology of both males and females. The principle of development of LHRH- based immunocontraceptive vaccines is based on observations that antibodies to LHRH block the action of the hormone on pituitary secretion of luteinising hormone and follicle stimulating hormone, leading to gonadal atrophy and sterility in mammals.

Most LHRH vaccines that have been developed consist of LHRH chemically conjugated to protein carriers to provide T cell help for the generation of anti-LHRH antibodies. It has been shown that upon repeated inoculation of LHRH-protein carrier conjugates the anti-LHRH titre decreases due to the phenomenon known as "carrier

induced epitope suppression". One aim of the present inventors is to replace protein carriers in the vaccines with defined T helper epitopes (TH-epitopes) so as to eliminate "carrier induced epitope suppression".

SUMMARY OF THE INVENTION

The present inventors have identified a number of 17 residue peptides each of which includes a T helper cell epitope. As will be readily appreciated the majority of these peptides are not minimal T helper cell epitopes. Typically class II molecules have been shown to be associated with peptides as short as 8 amino acids (Fahrer et al., 1995 ibid) but usually of 12-19 amino acids (Chicz, R. M., Urban, R. G., Gorga, J. C., Vignali, D. A. A., Lane, W. S. and Strominger, J. L. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. J Exp Med 1993, 178, 27-47; Chicz, R. M., Urban, R. G., Lane, W. S., Gorga, J. C., Stern, L. J., Vignali, D. A. A. and Strominger, J. L. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. Nature 1992, 358, 764-8), although, peptides up to 25 amino acids in length have been reported to bind to class II (reviewed in Rammensee, H.-G. Chemistry of peptide associated with class I and class II molecules. Curr Opin Immunol 1995, 7, 85-95.).

Thus peptide epitopes that range in length between 8 and 25 amino acid residues can bind to class II molecules. The shorter peptides are "core" epitopes that may have less activity than longer sequences but it is a trivial exercise to truncate longer sequences at the N- or the C-terminus to yield shorter sequences that have the same or better activity than the parent sequence.

Accordingly in a first aspect the present invention consists in a T helper cell epitope, the epitope being contained within a peptide sequence selected from the group consisting of SSKTQTHTQQDRPPQPS (SEQ ID NO: 1); QPSTELEETRTSRARHS (SEQ ID NO: 2); RHSTTSAQRSTHYDPRT (SEQ ID NO: 3); PRTSDRPVSYTMNRTRS (SEQ ID NO: 4); TRSRKQTSHRLKNIPVH (SEQ ID NO: 5); SHQYLVIKLIPNASLIE (SEQ ID NO: 6); IGTDNVHYKIMTRPSHQ (SEQ ID NO: 7); YKIMTRPSHQYLVIKLI (SEQ ID NO: 8); KLIPNASLIENCTKAEL (SEQ ID NO: 9); AELGEYEKLLNSVLEPI (SEQ ID NO: 10); KLLNSVLEPINQALTLM (SEQ ID NO: 11); EPINQALTLMTKNVKPL (SEQ ID NO: 12); FAGVVLAGVALGVATAA (SEQ ID NO: 13); GVALGVATAAQITAGIA (SEQ ID NO: 14); TAAQITAGIALHQSNLN (SEQ ID NO: 15); GIALHQSNLNAQAIQSL (SEQ ID NO: 16); NLNAQAIQSLRTSLEQS (SEQ ID NO: 17); QSLRTSLEQSNKAIEEI (SEQ ID NO: 18); EQSNKAIEEIREATQET (SEQ ID NO: 19); TELLSIFGPSLRDPISA (SEQ ID

NO: 20); PRYIATNGYLISNFDES (SEQ ID NO: 21); CIRGDTSSCARTLVSGT (SEQ ID NO: 22); DESSCVFVSESAICSQN (SEQ ID NO: 23); TSTIINQSPDKLLTFIA (SEQ ID NO: 24), SPDKLLTFIASDTCPLV (SEQ ID NO: 25) and SGRRQRRFAGVVLAGVA (SEQ ID NO: 26).

In a second aspect the present invention consists in a composition for use in raising an immune response in an animal, the composition comprising at least one T helper cell epitope, the at least one T helper cell epitope being contained within a peptide sequence selected from the group consisting of SSKTQTHTQQDRPPQPS (SEQ ID NO: 1); QPSTELEETRTSRARHS (SEQ ID NO: 2); RHSTTSAQRSTHYDPRT (SEQ ID NO: 3); PRTSDRPVSYTMNRTRS (SEQ ID NO: 4); TRSRKQTSHRLKNIPVH (SEQ ID NO: 5); SHQYLVIKLIPNASLIE (SEQ ID NO: 6); IGTDNVHYKIMTRPSHQ (SEQ ID NO: 7); YKIMTRPSHQYLVIKLI (SEQ ID NO: 8); KLIPNASLIENCTKAEL (SEQ ID NO: 9); AELGEYEKLLNSVLEPI (SEQ ID NO: 10); KLLNSVLEPINQALTLM (SEQ ID NO: 11); EPINQALTLMTKNVKPL (SEQ ID NO: 12); FAGVVLAGVALGVATAA (SEQ ID NO: 13); GVALGVATAAQITAGIA (SEQ ID NO: 14); TAAQITAGIALHQSNLN (SEQ ID NO: 15); GIALHQSNLNAQAIQSL (SEQ ID NO: 16); NLNAQAIQSLRTSLEQS (SEQ ID NO: 17); QSLRTSLEQSNKAIEEI (SEQ ID NO: 18); EQSNKAIEEIREATQET (SEQ ID NO: 19); TELLSIFGPSLRDPISA (SEQ ID NO: 20); PRYIATNGYLISNFDES (SEQ ID NO: 21); CIRGDTSSCARTLVSGT (SEQ ID NO: 22); DESSCVFVSESAICSQN (SEQ ID NO: 23); TSTIINQSPDKLLTFIA (SEQ ID NO: 24), SPDKLLTFIASDTCPLV (SEQ ID NO: 25) and SGRRQRRFAGVVLAGVA (SEQ ID NO: 26).

In a preferred embodiment of the present invention the composition comprises at least one peptide selected from the group consisting of SSKTQTHTQQDRPPQPS (SEQ ID NO: 1); QPSTELEETRTSRARHS (SEQ ID NO: 2); RHSTTSAQRSTHYDPRT (SEQ ID NO: 3); PRTSDRPVSYTMNRTRS (SEQ ID NO: 4); TRSRKQTSHRLKNIPVH (SEQ ID NO: 5); SHQYLVIKLIPNASLIE (SEQ ID NO: 6); IGTDNVHYKIMTRPSHQ (SEQ ID NO: 7); YKIMTRPSHQYLVIKLI (SEQ ID NO: 8); KLIPNASLIENCTKAEL (SEQ ID NO: 9); AELGEYEKLLNSVLEPI (SEQ ID NO: 10); KLLNSVLEPINQALTLM (SEQ ID NO: 11); EPINQALTLMTKNVKPL (SEQ ID NO: 12); FAGVVLAGVALGVATAA (SEQ ID NO: 13); GVALGVATAAQITAGIA (SEQ ID NO: 14); TAAQITAGIALHQSNLN (SEQ ID NO: 15); GIALHQSNLNAQAIQSL (SEQ ID NO: 16); NLNAQAIQSLRTSLEQS (SEQ ID NO: 17); QSLRTSLEQSNKAIEEI (SEQ ID NO: 18); EQSNKAIEEIREATQET (SEQ ID NO: 19); TELLSIFGPSLRDPISA (SEQ ID NO: 20); PRYIATNGYLISNFDES (SEQ ID NO: 21); CIRGDTSSCARTLVSGT (SEQ ID NO: 22); DESSCVFVSESAICSQN (SEQ

ID NO: 23); TSTIINQSPDKLLTFIA (SEQ ID NO: 24), SPDKLLTFIASDTCPLV (SEQ ID NO: 25) and SGRRQRRFAGVVLAGVA (SEQ ID NO: 26).

It is further preferred that the composition further comprises at least one B cell epitope and/or at least one CTL epitope.

In yet another preferred embodiment the at least one B cell epitope and/or the at least one CTL epitope are linked to at least one of the T helper cell epitopes. It is also preferred that the composition comprises a plurality of epitope constructs in which each comprises at least one T helper cell epitope and at least one B cell epitope. Alternatively the composition may comprises a plurality of epitope constructs in which each comprises at least one T helper cell epitope and at least one CTL epitope.

It will be understood that the B cell epitope or CTL epitope may be any epitope. A currently preferred B cell epitope is an LHRH B cell epitope.

The composition of the present invention may comprises a plurality of T helper cell epitopes. These epitopes may be singular or be linked together to form a single polypeptide. It will be understood that where the epitopes are linked to together in a single polypeptide the epitopes may be contiguous or spaced apart by additional amino acids which are not themselves part of the T helper cell epitopes.

As discussed above in one embodiment the T helper cell epitopes and at least one B cell epitope and/or at least one CTL epitope in which the epitopes are linked. This may be done by simple covalent linkage of the peptides. In another embodiment the epitopes are polymerised, most preferably such as described in PCT/AU98/00076, the disclosure of which is incorporated herein by reference.

In yet another preferred embodiment the composition further comprises a pharmaceutically acceptable excipient, preferably an adjuvant.

In a further aspect the present invention consists in a method of inducing an immune response in an animal, the method comprising administering to the animal the composition of the second aspect of the present invention.

Pharmaceutically acceptable carriers or diluents include those used in compositions suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. They are non-toxic to recipients at the dosages and concentrations employed. Representative examples of pharmaceutically acceptable carriers or diluents include, but are not limited to water, isotonic solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline) and can also contain one or more of, mannitol, lactose, trehalose, dextrose, glycerol, ethanol or polypeptides (such as human serum albumin). The

compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

As mentioned it is preferred that the composition includes an adjuvant. As will be understood an "adjuvant" means a composition comprised of one or more substances that enhances the immunogenicity and efficacy of a vaccine composition. Non-limiting examples of suitable adjuvants include squalane and squalene (or other oils of animal origin); block copolymers; detergents such as Tween®-80; Quil® A, mineral oils such as Drakeol or Marcol, vegetable oils such as peanut oil; *Corynebacterium*-derived adjuvants such as *Corynebacterium parvum*; *Propionibacterium*-derived adjuvants such as *Propionibacterium acne*; *Mycobacterium bovis* (Bacille Calmette and Guerin or BCG); interleukins such as interleukin 2 and interleukin 12; monokines such as interleukin 1; tumour necrosis factor; interferons such as gamma interferon; combinations such as saponin-aluminium hydroxide or Quil-A aluminium hydroxide; liposomes; ISCOM adjuvant; mycobacterial cell wall extract; synthetic glycopeptides such as muramyl dipeptides or other derivatives; Avridine; Lipid A derivatives; dextran sulfate; DEAE-Dextran or with aluminium phosphate; carboxypolymethylene such as Carbopol' EMA; acrylic copolymer emulsions such as Neocryl A640 (e.g. U.S. Pat. No. 5,047,238); vaccinia or animal poxvirus proteins; sub-viral particle adjuvants such as cholera toxin, or mixtures thereof.

As will be recognised by those skilled in the art modifications may be made to the peptides of the present invention without complete abrogation of biological activity. These modifications include additions, deletions and substitutions, in particular conservative substitutions. It is intended that peptides including such modifications which do not result in complete loss of activity as T helper cell epitopes are within the scope of the present invention.

Whilst the concept of substitution is well known in the field the types of substitutions envisaged are set out below.

Original Residue	Exemplary Substitutions	Preferred Substitutions
Ala (A)	val; leu; ile	Val
Arg (R)	lys; gln; asn	Lys
Asn (N)	gln; his; lys; arg	Gln
Asp (D)	glu	Glu
Cys (C)	ser	Ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro	pro
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe,norleucine	leu
Leu (L)	norleucine, ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile;	leu
Phe (F)	leu; val; ile; ala	leu
Pro (P)	Gly	gly
Ser (S)	Thr	thr
Thr (T	Ser	ser
Trp (W)	Tyr	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe ala; norleucine	leu

Another type of modification of the peptides envisaged include, but are not limited to, modifications to side chains, incorporation of unnatural amino acids and/or their derivatives during peptide synthesis and the use of crosslinkers and other methods which impose conformational constraints on the peptides.

Examples of side chain modifications contemplated by the present invention include, but are not limited to, modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidation with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2,4,6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5'-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid; 2-thienyl alanine and/or D-isomers of amino acids.

The peptides of the present invention may be derived from CDV. Alternatively, the peptide or combination of peptide epitopes may be produced by recombinant DNA technology. It is, however, preferred that the peptides are produced synthetically using methods well known in the field. For example, the peptides may be synthesised using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled "Peptide Synthesis" by Atherton and Sheppard which is included in a publication entitled "Synthetic Vaccines" edited by Nicholson and published by Blackwell Scientific Publications. Preferably a solid phase support is utilised which may be polystyrene gel beads wherein the polystyrene may be cross-linked with a small proportion of divinylbenzene (e.g. 1%) which is further swollen by lipophilic solvents such as dichloromethane or more polar solvents such as dimethylformamide (DMF). The polystyrene may be functionalised with chloromethyl or aminomethyl groups. Alternatively, cross-linked and functionalised polydimethyl-acrylamide gel is used which may be highly solvated and swollen by DMF and other dipolar aprotic solvents. Other supports can be utilised based on polyethylene glycol which is usually grafted or otherwise attached to the surface of inert polystyrene beads. In a preferred form, use may

be made of commercial solid supports or resins which are selected from PAL-PEG-PS, PAC-PEG-PS, KA, KR or TGR.

In solid state synthesis, use is made of reversible blocking groups which have the dual function of masking unwanted reactivity in the α-amino, carboxy or side chain functional groups and of destroying the dipolar character of amino acids and peptides which render them inactive. Such functional groups can be selected from t-butyl esters of the structure RCO-OCMe$_3$-CO. Use may also be made of the corresponding benzyl esters having the structure RCO-OCH$_2$-C$_6$H$_5$ and urethanes having the structure C$_6$H$_5$CH$_2$OCO-NHR which are known as the benzyloxycarbonyl or Z-derivatives and any Me$_3$-COCO-NHR, which are known as t-butoxyl carbonyl, or Boc derivatives. Use may also be made of derivatives of fluorenyl methanol and especially the fluorenyl-methoxy carbonyl or Fmoc group. Each of these types of protecting group is capable of independent cleavage in the presence of one other so that frequent use is made, for example, of BOC-benzyl and Fmoc-tertiary butyl protection strategies.

Reference also should be made to a condensing agent to link the amino and carboxy groups of protected amino acids or peptides. This may be done by activating the carboxy group so that it reacts spontaneously with a free primary or secondary amine. Activated esters such as those derived from p-nitrophenol and pentafluorophenol may be used for this purpose. Their reactivity may be increased by addition of catalysts such as 1-hydroxybenzotriazole. Esters of triazine DHBT (as discussed on page 215-216 of the abovementioned Nicholson reference) also may be used. Other acylating species are formed in situ by treatment of the carboxylic acid (i.e. the N-alpha-protected amino acid or peptide) with a condensing reagent and are reacted immediately with the amino component (the carboxy or C-protected amino acid or peptide). Dicyclohexylcarbodiimide, the BOP reagent (referred to on page 216 of the Nicholson reference), O'Benzotriazole-N, N, N'N'-tetra methyl-uronium hexafluorophosphate (HBTU) and its analogous tetrafluoroborate are frequently used condensing agents.

The attachment of the first amino acid to the solid phase support may be carried out using BOC-amino acids in any suitable manner. In one method BOC amino acids are attached to chloromethyl resin by warming the triethyl ammonium salts with the resin. Fmoc-amino acids may be coupled to the p-alkoxybenzyl alcohol resin in similar manner. Alternatively, use may be made of various linkage agents or "handles" to join the first amino acid to the resin. In this regard, p-hydroxymethyl phenylacetic acid linked to aminomethyl polystyrene may be used for this purpose.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated

element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

DETAILED DESCRIPTION OF THE INVENTION

In order that the nature of the present invention may be more readily understood preferred forms there of will now be described with reference to the following non-limiting examples.

FIGURE LEGENDS

Figure 1. Amino acid sequence of the fusion protein of CDV (SEQ ID NO: 27)

Figure 2a. Stimulation indices to Th-epitope P25 and its truncated versions in Dog #70 immunised with P25-LHRH (X-axis concentration of peptides nmoles/well).

Figure 2b Stimulation indices to Th-epitope P25 and its truncated versions in Dog #73 immunised with P25-LHRH (X-axis concentration of peptides nmoles/well).

Figure 2c Stimulation indices to Th-epitope P25 and its truncated versions in Dog #127 immunised with P25-LHRH (X-axis concentration of peptides nmoles/well).

Figure 2d Stimulation indices to Th-epitope P25 and its truncated versions in Dog #993 immunised with P25-LHRH (X-axis concentration of peptides nmoles/well).

Figure 3a. Stimulation indices to Th-epitope P27 and its truncated 15-mer in Dog #105 immunised with P27-LHRH. (X-axis concentration of peptides nmoles/well).

Figure 3b. Stimulation indices to Th-epitope P27 and its truncated 15-mer in) Dog #94 immunised with P27-LHRH. (X-axis concentration of peptides nmoles/well).

Figure 3c. Stimulation indices to Th-epitope P27 and its truncated 15-mer in Dog #20 immunised with P27-LHRH. (X-axis concentration of peptides nmoles/well).

Figure 3d. Stimulation indices to Th-epitope P27 and its truncated 15-mer in Dog #101 immunised with P27-LHRH. (X-axis concentration of peptides nmoles/well).

Figure 4a. Stimulation indices to Th-epitope P35 and its truncated versions in Dog #19 immunised with P35-LHRH (X-axis concentration of peptides nmoles/well).

Figure 4b. Stimulation indices to Th-epitope P35 and its truncated versions in Dog #100 immunised with P35-LHRH (X-axis concentration of peptides nmoles/well).

Figure 4c. Stimulation indices to Th-epitope P35 and its truncated versions in Dog #96 immunised with P35-LHRH (X-axis concentration of peptides nmoles/well).

Figure 4d. Stimulation indices to Th-epitope P35 and its truncated versions in Dog #102 immunised with P35-LHRH (X-axis concentration of peptides nmoles/well).

EXAMPLE 1

Identification of T helper cell epitopes

Methods and Results:

Towards identification of canine T cell epitopes 94, 17 residue overlapping peptides were designed encompassing the entire sequence of fusion protein of canine distemper virus (CDV). The 17mer peptides were numbered sequentially for identification starting from the N-terminus. The sequence of the fusion protein of CDV as determined by Barrett *et al* 1987 (Virus Res. 8, 373-386) is shown in Figure 1. The peptides were used in T-cell proliferation assays using peripheral blood lymphocytes (PBMC) from dogs immunised with Canvac™ 3 in 1 vaccine (CSL Limited) which contains live CDV.

Initially, four dogs were used and they were boosted with the Canvac™ 3 in 1 vaccine twice with four to six weeks between each vaccination. The dogs were bled after each booster vaccination and the PBMCs were tested against the peptides. No significant proliferation to peptides was observed.

Since CDV has been reported to be lymphotropic and the vaccine consists of live CDV, there was the possibility that it may be sequestered in lymphoid organs preventing significant numbers of precursor T cells entering the peripheral system. To increase the frequency of peripheral blood anti-CDV T cells dogs were boosted with heat killed CDV (obtained as a pellet from virus culture medium, CSL Limited). Two weeks later, the dogs were bled and the PBMCs tested for proliferation against the peptides. Again there was no proliferation to the peptide antigens.

An alternate strategy was used to increase the precursor frequency of specific T cells recognising the CDV peptides. Fresh PBMCs obtained from these hyperimmunised dogs were subjected to stimulation *in vitro* with pools of all 94 peptides for 30 minutes at 37^0C. The cells were then washed to remove any excess peptides and cultured for 7 days. This population of T cells was then tested with autologous APCs with every single peptide as the antigen. Table 1 shows the peptides to which significant (stimulation index >2) levels of proliferation were observed.

To confirm this observation, the same four dogs were bled again, five weeks after receiving the dose of killed virus. The PBMCs were stimulated *in vitro* with pools of either all 94 peptides or peptides 21-40 (because most of the activity was in this region) and after 7 days of culture the stimulated T cells were tested against individual peptides. Significant stimulatory indices were obtained with all peptides, confirming the above results. Four more dogs which received only one dose of 3 in 1 vaccine were tested using the *in vitro* stimulation method and all four dogs responded to the majority of peptides shown in Table 2.

The above peptides were also tested on cells from additional dogs, with results shown in Table 3. Peptides P64, P74 and P75 were also shown to react strongly with peripheral blood mononuclear cells from dogs of various breeds immunised with CDV (Table 4), and are therefore identified as strong T-helper epitopes.

Table 1.
Identification of canine T cell epitopes from the sequence of fusion protein of CDV.

Peptides	Beagle Foxhound (Dog#18)	Beagle Foxhound (Dog#19)	Beagle Foxhound (Dog#20)	Beagle Foxhound (Dog#21)
p2	2*	<2	8	3.9
p4	4.9	<2	3.3	4.6
p6	2.5	<2	4	5.1
p10	2.3	<2	3.2	9.1
p24	5.8	9.9	2.8	29
p25	3.2	11.9	4.5	17
p27	3.3	34	6.7	14.8
p29	3.5	42	4.4	<2
p35	3.1	57	3.3	22
p36	6.7	3.7	3.3	16
p37	6.9	10.9	8.2	26
p38	2.8	6.7	3.6	4.2
p47	3.3	85.7	2.9	1.9
p62	<2	51	5.6	4.2
p68	6.6	<2	<2	11.7

*Stimulatory index

Table 2.
Identification of canine T cell epitopes from the sequence of fusion protein of CDV.

Peptides	Beagle Foxhound (Dog #70)	Beagle Foxhound (Dog #71)	Beagle Foxhound (Dog #72)	Beagle Foxhound (Dog #73)
p8	2.2			
p22			2.6	
p24		3.2	2.2	
p25	1.5	2.9	2	12
p27		2.7	3.5	4.8
p28		2		
p29		2		6
p33			1.6	
p35			1.7	6.8
p37			1.7	
p62		3		

Table 3.
Identification of canine T cell epitopes from the sequence of fusion protein of CDV.

Peptides	Kelpie Foxhound (Dog#125)	Kelpie Foxhound (Dog#126)
p23	3.2	
p27	4.5	8.5
p28	1.9	
p29	3.6	
p33	6	
p34	2.1	
p35	3.8	10
p36	3	
p37	2.5	
p38	2.2	
p39	2.9	
p47	2.7	
p62	2.4	
p68	2.9	

Table 4.
Identification of canine T cell epitopes from the sequence of fusion protein of CDV.

Peptides	Poodle Shitzu	Beagle Foxhound#18	Beagle Foxhound#19	Beagle Foxhound#20	Beagle Foxhound#21
P64	50.0		2.5	2.5	
P74	4.0			1.7	6.0
P75	10	2.5			7.2

Once again the same peptides and one additional peptide P32 were tested on cells from additional dogs. These peptides were also shown to react strongly with peripheral blood mononuclear cells from dogs of various breeds immunised with CDV (Table 5), and are therefore identified as strong T-helper epitopes.

In conclusion, 26 peptides were identified as canine T helper cell epitopes in the fusion protein of CDV. The sequences of each of these peptides are set out in Table 6.

These T helper cell epitopes will have usefulness as components of animal, in particular, canine vaccines, either simply as synthetic peptide based vaccines and as additions to vaccines containing more complex antigens.

Table 5.
Identification of canine T cell epitopes from the sequence of fusion protein of CDV.

Peptides	Poodle Shitzu	Grey hound	Fox Terrier	Terrier Cross	Kelpie Pointer	Border Collie
P2	140	<2	<2	<2	2.6	2
P4	44	2	<2	2	3.5	2
P6	38	<2	<2	<2	<2	2
P8	100	2	<2	<2	2.8	2
P10	50	2	2.2	2.1	2.4	3
P25	<2	<2	2.6	<2	2.6	<2
P29	2	<2	<2	2	<2	<2
P32	<2	2	<2	<2	<2	<2
P33	<2	<2	<2	<2	2	2
P35	<2	<2	2.2	<2	2	2
P37	<2	<2	<2	2	2	<2
P62	24	<2	<2	<2	<2	<2
P64	50	<2	<2	<2	<2	<2
P68	5	<2	<2	<2	<2	<2
P74	4	<2	<2	<2	<2	<2
P75	10	<2	<2	<2	<2	<2

Table 6.

Sequences of the peptides:

P2	SSKTQTHTQQDRPPQPS (SEQ ID NO: 1)
P4	QPSTELEETRTSRARHS (SEQ ID NO: 2)
P6	RHSTTSAQRSTHYDPRT (SEQ ID NO: 3)
P8	PRTSDRPVSYTMNRTRS (SEQ ID NO: 4)
P10	TRSRKQTSHRLKNIPVH (SEQ ID NO: 5)
P24	SHQYLVIKLIPNASLIE (SEQ ID NO: 6)
P22	IGTDNVHYKIMTRPSHQ (SEQ ID NO: 7)
P23	YKIMTRPSHQYLVIKLI (SEQ ID NO: 8)
P25	KLIPNASLIENCTKAEL (SEQ ID NO: 9)
P27	AELGEYEKLLNSVLEPI (SEQ ID NO: 10)
P28	KLLNSVLEPINQALTLM (SEQ ID NO: 11)
P29	EPINQALTLMTKNVKPL (SEQ ID NO: 12)
P32	SGRRQRRFAGVVLAGVA (SEQ ID NO: 26)
P33	FAGVVLAGVALGVATAA (SEQ ID NO: 13)
P34	GVALGVATAAQITAGIA (SEQ ID NO: 14)
P35	TAAQITAGIALHQSNLN (SEQ ID NO: 15)
P36	GIALHQSNLNAQAIQSL (SEQ ID NO: 16)
P37	NLNAQAIQSLRTSLEQS (SEQ ID NO: 17)
P38	QSLRTSLEQSNKAIEEI (SEQ ID NO: 18)
P39	EQSNKAIEEIREATQET (SEQ ID NO: 19)
P47	TELLSIFGPSLRDPISA (SEQ ID NO: 20)
P62	PRYIATNGYLISNFDES (SEQ ID NO: 21)
P68	CIRGDTSSCARTLVSGT (SEQ ID NO: 22)
P64	DESSCVFVSESAICSQN (SEQ ID NO: 23)
P74	TSTIINQSPDKLLTFIA (SEQ ID NO: 24)
P75	SPDKLLTFIASDTCPLV (SEQ ID NO: 25)

Selected sequences of the identified T-cell epitopes were tested for their ability to induce an antibody response to a linked B-cell epitope. Trials were conducted in dogs for assessment of antibody responses. The T–cell epitopes were linked to the B cell epitope LHRH (leuteinising hormone releasing hormone), with the T-cell epitope at the N-terminus and LHRH positioned at the carboxy terminus.

Peptides were synthesised using standard chemistry with Fmoc protection. All peptides were purified to at least 80% purity and the product checked by mass spectroscopy.

The peptides were produced as contiguous T-cell – B cell determinants. The LHRH sequence of Pyro Glu –His –Trp – Ser – Tyr – Gly – Leu – Arg – Pro – Gly (SEQ ID NO: 28), or variations of it, was linked to the carboxyl terminus of each respective CDV T- helper epitope.

In-vivo evaluation of some of the T-helper epitopes was conducted in two trials, by vaccination of dogs with T-helper – LHRH sequences.

EXAMPLE 2. (Trial K9-5)

A total of 14 dogs of mixed sex were used in this trial. All had been previously vaccinated with a live CDV vaccine and had also been vaccinated against LHRH.

Vaccine formulation.

Test peptides P25, P27, P35 from CDV were synthesised with LHRH at the C terminus of each T-helper epitope. The LHRH sequence used was the full 10 amino acids of the native LHRH. Each of the vaccine constructs, together with a control peptide comprising a mouse influenza T-cell epitope linked to a repeat malarial B-cell epitope (sequence shown in table below) were purified to ~80-90% purity. All peptides were dissolved in 4M urea before dilution with sterile saline to an appropriate volume to give 40nmoles per 1 mL dose. Iscomatrix™ was added to a final concentration of 150ug / 1 mL dose as adjuvant together with thiomersal preservative (0.01%).

ISCOM™ or Immunostimulating Complex (Barr, Sjolander and Cox, 1998, Advanced Drug Delivery Systems 32: 247 – 271) are a well characterised class of adjuvant comprised of a complex of phospholipid, cholesterol and saponin, usually with a protein incorporated into the complex. Where the complex is formed in the absence of protein antigen, then this complex is termed Iscomatrix™. The saponin used in the preparation of this adjuvant was Quil A.

Vaccination, blood samples and assays.

All dogs were vaccinated with a 1mL dose, delivered in the scruff of the neck. Vaccinations were given at 0 and 4 weeks and venous blood samples were obtained at intervals during the trial.

Effective T-cell help was determined by measuring the antibody response to LHRH by ELISA. Biological effectiveness of the peptide based vaccine was determined by measuring the levels of progesterone in female dogs and testosterone in male dogs.

Table 7.

Trial Groups

Peptide	Dog Nos.
Control –ALNNRFQIKGVELKS –(NANP)3 (SEQ ID NO: 30)	104, 998
P25 –LHRH 1-10	70, 73, 127, 993
P27 –LHRH 1-10	20, 94, 101, 105
P35 –LHRH 1-10	19, 96, 100, 102

Results

Pre-existing low antibody levels to LHRH were present in all dogs due to immunisation previously with a different vaccine. The control group of dogs exhibited a slow decrease in antibody levels.

Dogs immunised with P25-LHRH, P27-LHRH and P35-LHRH all showed strong antibody responses to the B-cell epitope (LHRH). This response persisted to 6 weeks post boost vaccination (see Table 8).

The biological potency of the vaccine was demonstrated by a significant reduction in progesterone or testosterone levels (see Tables 9 and 10).

Table 8
Anti LHRH Titres

		Anti LHRH Titres		
Peptide	Dog No	Prebleeds	2 wks post boost	6wks post boost
Control	104	1258	1975	1936
	998	2559	1982	1947
Average		1794	1978	1941
Range		1258-2559	1975-1982	1936-1947
P25-LHRH (1-10)	70	856	24245	16697
	73		42665	16922
	127	1361	21485	19662
	993	577	24879	15119
Average		886	23120	17242
Range		0 -1361	21485-42665	15119-19662
p27-LHRH (1-10)	20	747	29653	8423
	94		41247	22759
	101	4256	52724	17353
	105	944	12600	8366
Average		2004	25774	12049
Range		747-4256	12600-52724	8366-22759
p35-LHRH (1-10)	19	665	18033	6228
	96	1621	26583	5744
	100	580	17255	4829
	102	180	11740	2963
Average		323	14233	3783
Range		180-1621	11740-26583	2963-6228

Table 9.

Progesterone results (nmol/L)

Peptide	Dog No.	4wks post primary	2wks post boost	6wks post boost
Control	998	5.17	4.28	<0
p25-LHRH (1-10)	127	3.04	4.83	<0
	993	1.7	0.87	<0
p27-LHRH (1-10)	101	0.42	0.14	<0
p35-LHRH (1-10)	96	31.76	2.15	<0
	100	<0	<0	<0

Table 10.

Testosterone results (nmol/L)

Peptide	Dog No.	4wks post primary	2wks post boost	6wks post boost
Control	104	9.69	2.51	3.31
p25-LHRH (1-10)	70	<0	<0	<0
	73	5.38	<0	<0
p27-LHRH (1-10)	20	1.04	<0	<0
	94	3.33	<0	<0
	105	>47.7	<0	<0
p35-LHRH (1-10)	19	4.3	2.77	4.55
	102	6.72	<0	<0

The effectiveness of selected T-cell epitopes from the F-protein of CDV in providing T-cell help to elicit antibody responses in dogs proves that the identified sequences are functional. These results also validate the scientific approach and usefulness of the *in vitro* screening method for identifying T-helper epitope sequences with *in vivo* activity.

EXAMPLE 3 (Trial K9-8)

A total of 35 dogs mixed sex were used in this trial. All had been previously vaccinated with a live CDV vaccine but had not been vaccinated against LHRH.

Vaccine Formulation:

The T-Helper epitopes were linked to a truncated form of LHRH, containing amino acids 2 to 10 of the native 10 amino acid sequence , as shown below:

2-10 LHRH His –Trp – Ser – Tyr – Gly – Leu – Arg – Pro – Gly (SEQ ID NO: 29).

All vaccines were formulated as for Example 2, ie each 1 mL dose of vaccine contained 40nmoles of peptide, 150μg Iscomatrix™, and thiomersal as preservative.

Where dogs were vaccinated with a pool of peptides, the concentration of each peptide was adjusted to give equal concentrations and a total amount of 40nmoles of LHRH epitope per 1 mL dose.

Vaccination, blood samples and assays.

All dogs were vaccinated with a 1mL dose, delivered in the scruff of the neck. Vaccinations were given at 0 and 4 weeks and venous blood samples were obtained at intervals during the trial.

Effective T-cell help was determined by measuring the antibody response to LHRH by ELISA. Biological effectiveness of the peptide based vaccine was determined by measuring the levels of progesterone in female dogs and testosterone in male dogs.

Table 11.
Trial Groups

Peptide Group	Dog Nos.
P25–LHRH 2-10	211, 195, 197, 181
P27–LHRH 2-10	203, 191, 186, 201
P35–LHRH 2-10	217, 198, 187, 196
Pool: P25–LHRH 2-10, P27–LHRH 2-10, P35–LHRH 2-10	212, 193, 178, 216, Y3
P2–LHRH 2-10	194, 199, 179, 220
P8–LHRH 2-10	Y4, Y6, 160, 200
P62–LHRH 2-10	219, 185, 221, 177
P75–LHRH 2-10	189, 222, 202, 176
Unvaccinated controls	190, 159

Results

Strong antibody responses to LHRH were demonstrated in dogs immunised with the T-cell-LHRH constructs with the T-cell epitopes P25, P27, P35, P62, P75, and the pool of T-cell-LHRH peptides comprising a combination of T-cell epitopes P25, P27 and P35 (see Table 12).

Low to undetectable antibody responses were seen in dogs immunised with P2 and P8-LHRH peptides (see Table 12). This was concluded to indicate that these T-cell peptides were not well recognised by Beagle-Foxhound dogs, which is consistent with their identification using PBMCs' from other dog breeds. The initial screening in Beagle foxhound dogs indicated that this breed of dog does not respond to these 2 T-cell epitopes.

As is well understood by those skilled in the art of peptide vaccines the response to individual peptides is genetically determined. The class II Major Histocompatability Complex (MHC II) is polymorphic. Class II molecules at the cell surface function to bind peptides for presentation to T-cells, which is required as part of the activation process for T-cells, including helper T-cells. The allelic forms of MHC class II bind discrete sets of peptide antigens, and thus the response to those antigens is genetically determined. Thus the results are interpreted to indicate that the Beagle – Foxhound breed of dog does not possess the appropriate MHC-II alleles to respond to P2 and P8, but that

other breeds of dog do, eg. the Poodle Shitzu breed that were used to identify these peptides.

Control dogs showed no change in antibody levels to LHRH during the trial period and hormone levels were within normal ranges for the age and sex of the dogs (see Table 12).

Table 12

Anti-LHRH Titres

			Anti LHRH Titres		
Peptide	Group	Dog No	4 wks after primary	2 wks post boost	4 wks post boost
Control	1	159	0	0	0
	1	190	0	0	0
	GMT				
Pool	2	Y3	1860	55659	95038
	2	178	17900	416036	486793
	2	193	8770	211369	189143
	2	212	3766	121411	135293
	2	216	8378	294769	642293
	GMT		6207	177292	237798
P25-LHRH	3	181	1893	152264	131643
	3	195	31197	205906	455193
	3	197	14423	337698	240543
	3	211	20607	142798	131643
	GMT		11510	193037	214229
P27-LHRH	4	186	0	11206	17263
	4	191	0	59154	125493
	4	201	0	17041	34103
	4	203	0	1000	857
	GMT		0	18523	26698
P35-LHRH	5	187	2009	141775	55797
	5	196	4868	237208	158040
	5	198	1539	154375	68307
	5	217	0	121050	40822
	GMT		2469	103085	58002

Table 12
Anti-LHRH Titres (Cont.)

			Anti LHRH Titres		
Peptide	Group	Dog No	4 wks after primary	2 wks post boost	4 wks post boost
P2-LHRH	6	179	0	0	0
	6	194	0	0	0
	6	199	0	0	0
	6	220	0	0	0
	GMT				
P8-LHRH	7	Y4	0	0	0
	7	Y6	0	0	0
	7	160	0	1200	ND
	7	200	0	8000	2227
	GMT				
P62-LHRH	8	177	1242	3821	2985
	8	185	0	146581	67461
	8	219	0	29353	28282
	8	221	2697	231473	156549
	GMT		1830	44167	30728
P75-LHRH	9	176	0	12177	5559
	9	189	0	15795	17155
	9	202	0	2121	2216
	9	222	0	9787	7879
	GMT			11201	8746

EXAMPLE 4

In vitro T cell Proliferation Assays to demonstrate recognition of Th-epitope incorporated in the peptide vaccines

To demonstrate recognition of the Th-epitope within the peptide immunogen PBMCs obtained from dogs immunised with peptide vaccines (dogs from Example 2) were tested against the respective Th-epitopes. The assay was carried out without the enrichment of PBMCs. PBMCs obtained from Ficoll gradient purification were directly tested against the respective Th-epitope and its truncated versions. The study

demonstrated that all the dogs immunised with peptide vaccines responded to the Th-epitope incorporated confirming that T- cell activity resides in the respective sequences (Figures 2-4). Truncated versions of the respective Th-epitopes were also tested to more closely define the T-cell activity within the sequences. It was observed that for P25 the full sequence of 17 residues was better than the shorter peptides of 15 and 12 residues, each truncated from the N-terminus of the sequence (Figure 2). This implies that the T-cell activity is towards the N-terminus or middle of the 17-residue peptide.

A similar observation was made with P27, the 17 residue long peptide was a better simulator than the 15-mer truncated from the N-terminus (Figure 3). This observation again suggested that the T-cell activity may reside towards the middle or the N-terminus of the full length peptide.

In the case of P35 and its shorter versions, except for one dog (#102), the other three dogs responded as well to the 12 residue peptide as to the full length 17 residue one (Figure 4). In dog # 102 the 15 residue peptide was more stimulatory than the full length peptide. From this it can be deduced that that the first two residues in the sequence of P35 may not be essential and that the activity is towards the middle or C-terminus of the peptide.

EXAMPLE 5

Trial in BALB/c mice

The canine vaccines with CDV-F derived Th-epitopes and LHRH used in Example 3 were also used to immunise BALB/c mice to investigate if the Th-epitopes would be functional in a different animal species.

Vaccine Formulation

All vaccines were formulated as for Example 3 except that they were diluted further so that 100μl doses contained 2.7 nmoles of peptide and 10μg of Iscomatrix™ and thiomersol as preservative.

Vaccination, blood samples and assays.

Mice were vaccinated with 100μl of the vaccine at the base of tail. Vaccinations were given at 0 and 4 weeks and animals bled at intervals after each vaccination from the retro-orbital plexus. Effective T-cell help was determined by measuring the antibody response to LHRH by ELISA.

Results

Mice immunised with P25-LHRH and pool of peptides comprising of P25-LHRH, P27-LHRH and P35-LHRH generated high antibody titres to LHRH. Peptides P35 and P75 generated low antibody titres whereas mice immunised P2, P8 and P62 had undetectable levels of anti-LHRH antibodies (Table 13).

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Table 13.

Anti-LHRH antibody titres in mice immunised with CDV-F derived T cell epitope-LHRH vaccines

Groups	4 weeks post first vaccination					2 weeks post second vaccination				
	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5
Group 1(control)	<100	<100				<100	<100			
Group 2 (pool)	100	126	200	126	200	16,000	16,000	16,000	16,000	16,000
Group 3 (p25-LHRH)	126	400	282	100	282	16,000	16,000	16,000	16,000	16,000
Group 5 (p35-LHRH)	<100	<100	<100	<100	<100	1,412	800	<100	<100	<100
Group 6 (p2-LHRH)	<100	<100				<100	<100			
Group 7 (p8-LHRH	<100	<100	<100	<100		<100	<100	<100	<100	
Group 8 (p62-LHRH	<100	<100	<100			126	126	<100		
Group 9 (p75-LHRH	<100	<100	<100	<100	<100	<100	<100	316	3,162	<100